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**ELECTROCHEMILUMINESCENCE FROM
TUNICATE, TUNICHRONE—METAL
COMPLEXES AND OTHER BIOLOGICAL
SAMPLES**

POSTPRINT

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14. ABSTRACT Low level intrinsic electrochemiluminescence (ECL) was induced from body fluids and homogenized tissues of oysters and several species of tunicates. No significant ECL was detected in human blood cell lysates, or bovine haematin, but minor ECL was observed in avian blood cell lysates. Both terrestrial grass and seagrass exhibited ECL, which is probably attributable to chlorophyll, since dead (brown) grass did not demonstrate ECL. It was postulated that organic-metal complexes in marine invertebrates were, at least in part, responsible for the intrinsic ECL, since such animals are known to be rich in organically bound metals. However, alternative biochemical mechanisms for the observed ECL, which do not involve metal chelates, are possible. Various metal ions were added to the invertebrate preparations to determine whether exogenous metals could enhance or inhibit the ECL reactions. Strongly oxidizing metal ions such as Ag^+ , Au^+ , Cu^{2+} , Hg^{2+} and Sb^{2+} at ≥ 100 ppm severely inhibited the intrinsic ECL response. No statistically significant ECL enhancement due to addition of metal ions was noted. ECL 'profiles' were generated which demonstrated differences in the ECL responses of individual tunicate preparations to the presence of various exogenous metal ions. Differences in ECL profiles may represent differences in types or levels of endogenous metal chelates or other biochemical constituents. In addition, synthetic tunichromes (tunicate pigments) were analysed for ECL in the presence and absence of various added metal ions. One synthetic tunichrome isomer demonstrated a specific ECL interaction with Hg^{2+} , while the other demonstrated broader ECL activity with several metal ions.					
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Electrochemiluminescence from Tunicate, Tunichrome–Metal Complexes and Other Biological Samples

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Low level intrinsic electrochemiluminescence (ECL) was induced from body fluids and homogenized tissues of oysters and several species of tunicates. No significant ECL was detected in human blood cell lysates, or bovine haematin, but minor ECL was observed in avian blood cell lysates. Both terrestrial grass and seagrass exhibited ECL, which is probably attributable to chlorophyll, since dead (brown) grass did not demonstrate ECL. It was postulated that organic–metal complexes in marine invertebrates were, at least in part, responsible for the intrinsic ECL, since such animals are known to be rich in organically bound metals. However, alternative biochemical mechanisms for the observed ECL, which do not involve metal chelates, are possible. Various metal ions were added to the invertebrate preparations to determine whether exogenous metals could enhance or inhibit the ECL reactions. Strongly oxidizing metal ions such as Ag^+ , Au^+ , Cu^{2+} , Hg^{2+} and Sb^{2+} at ≥ 100 ppm severely inhibited the intrinsic ECL response. No statistically significant ECL enhancement due to addition of metal ions was noted. ECL 'profiles' were generated which demonstrated differences in the ECL responses of individual tunicate preparations to the presence of various exogenous metal ions. Differences in ECL profiles may represent differences in types or levels of endogenous metal chelates or other biochemical constituents. In addition, synthetic tunichromes (tunicate pigments) were analysed for ECL in the presence and absence of various added metal ions. One synthetic tunichrome isomer demonstrated a specific ECL interaction with Hg^{2+} , while the other demonstrated broader ECL activity with several metal ions.

Keywords: electrochemiluminescence; metals; tunicates; molluscs; plants

INTRODUCTION

Electrochemiluminescence (ECL) has been described for several different types of molecular complexes including purely organic, organic–metal chelates and metallic halogen 'clusters' (1–3).

In recent years, ECL has been employed in a number of very sensitive immunoassays and nucleic acid-based assays (4–10). The body of literature on ECL involves primarily synthetic ECL coordination complexes such as ruthenium (II) trisbipyridine ($\text{Ru}(\text{bpy})_3^{2+}$), Cr, Cu, Eu, Ir, Os, Pd, Pt, Re and other metal chelates (1–3, 11, 12). Ultimately, however, it might be of commercial

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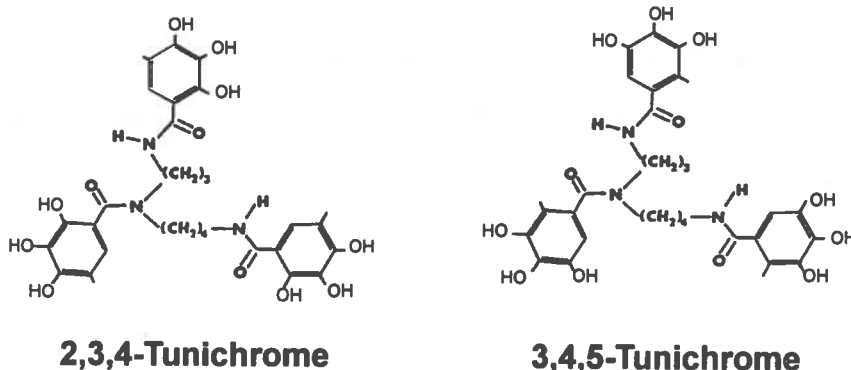


Figure 1. Chemical structures of synthetic tunichromes used in these studies (15)

value to identify abundant, inexpensive and easily extractable natural sources of ECL compounds or chelating ligands that exhibit ECL upon binding certain metals, for use in a variety of ECL-based assays and metal biosensors. The present work describes relatively low level, but detectable biological ECL from certain types of animals and plants.

It was hypothesized that certain naturally occurring organic-metal chelates which utilize aminoaromatic ligands might exhibit detectable ECL, because of structural similarity to $\text{Ru}(\text{bpy})_3^{2+}$ and other ECL compounds. One such class of metal chelators may be the 'tunichromes' (Fig. 1; 13–20) or tunicate blood cell pigments. Alternatively, tunichromes may act only as reducing agents for metals, while semiquinones (20,21), tunichlorins (14) and cyclic peptides such as the lissoclinamides (14,22) may form the actual ECL complexes with metals.

Tunicates are sessile marine invertebrates, commonly referred to as 'sea-squirts' or ascidians, which accrue certain metals, most notably Fe and V, by as much as 10^7 -fold over ambient concentrations or up to 1 M in certain tissues (14,19,21). Evidence suggests that tunicate accumulation of metals may be largely mediated by certain types of tunicate blood cells, which fluoresce brightly (23,24) and are thought to contain tunichromes (16,17,23,25). Organic-metal complexes may also exist cell-free in tunicate body fluids (25, 26). Therefore, in attempting to identify sources of biological ECL, tunicates and tunicate body fluids seemed logical starting points.

In certain species of tunicates, especially small colonial varieties, collection of blood or body fluid samples is difficult, thus cleaned whole animal homogenates were prepared for ECL analyses.

Molluscs have also been noted for their metal accrual (14,27). Therefore, oyster homogenate were also examined for intrinsic ECL. In addition isolated haematin, human and avian blood cell lysates, phytoplankton and plant preparation were examined for possible ECL of metallo-porphyrins such as haeme and chlorophylls (28–30).

ECL was measured in a commercially available instrument designed to measure $\text{Ru}(\text{bpy})_3^{2+}$ -based ECL by utilizing tripropylamine (TPA) as an electron carrier for the redox reaction (4–6,11). It was thought that the intrinsic tunicate ECL compound might be an organic-metal coordination complex because tunicates are rich in metals and potential organic metal chelators (13–20). However, the possibility remains that the intrinsic ECL molecule are not metallic coordination complexes, but are purely organic (e.g. polyaromatic hydrocarbon or luminol-like molecules), metal-nonmetal 'clusters' (such as the Mo and W chloride clusters), or some unknown class of biochemical (1,2). Finally, synthetic tunichrome analogues (15) were examined for ECL in the presence and absence of a variety of metal ions to determine whether tunichromes could form ECL complexes with metals or emit ECL in isolation.

Fig. 2 (pathway I) illustrates the basic postulated mechanism of intrinsic ECL that can be generated from tunicate or other biological sources using the commercial ECL sensor. Pathway I involves the generation of a high-energy cationic free radical form of TPA ($\text{TPA}^{+\bullet}$) following its oxidation at an anode. The radical electron from $\text{TPA}^{+\bullet}$ can be transferred to an oxidized endogenous metal coordination complex or other ECL compound. When the transferred electron relaxes to a ground state, a photon is emitted (1–4). It was

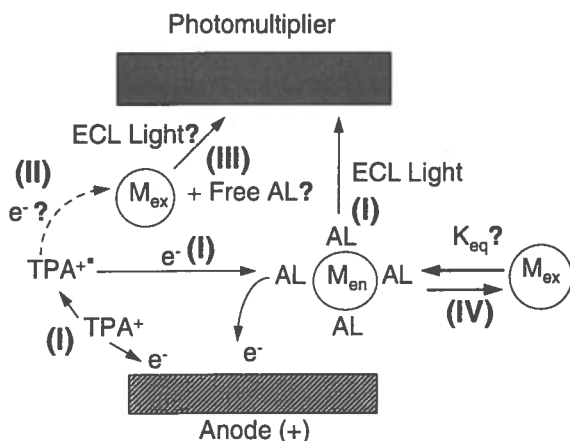


Figure 2. Generalized model of possible organic-metal biological ECL reactions occurring in the ECL sensor. Purely organic or other types of compounds may also be responsible for the observed ECL. Roman numerals in parentheses indicate reaction pathways delineated in the text. M_{ex} = exogenous metal, M_{en} = endogenous metal, AL = aromatic ligand, TPA = tripropylamine

hypothesized that the addition of exogenous metal ions might inhibit intrinsic biological ECL by usurping electrons from $\text{TPA}^{+\bullet}$ (Fig. 2; pathway II) or might enhance intrinsic ECL by binding to free aromatic ligands (pathway III), or replacing resident metals in existing metal coordination complexes (pathway IV).

MATERIALS AND METHODS

Samples and sample preparation

Two species of relatively large (several cm in length and width) solitary tunicates (*Molgula occidentalis* and *Stryela plicata*) and one reddish colonial tunicate (*Diplosoma macdonaldi*) were collected during autumn from diverse locations in St Andrew Sound in north-west Florida. Both fresh and frozen animals were analysed. Fresh animals were sometimes kept in static aquaria for several days prior to processing.

The exterior tunics of *M. occidentalis* specimens were scrubbed in sea water to remove debris and a small longitudinal incision was made in the tunic with a razor blade below the atrial siphon. The animals were exsanguinated by squeezing gently and several ml of light brown body fluid (pH 6.4) were collected in sterile plastic petri dishes.

Some frozen and once-thawed *M. occidentalis* blood cells were ruptured in deionized water containing 0.5% Triton X-100 for ≥ 30 min at room temperature (RT) in order to examine the effect of cell lysis on ECL. Body fluids were diluted as appropriate to produce roughly equal absorbances at 600 nm ($A_{600\text{nm}} \cong 0.33$) for comparison of individual animals or samples. Unfortunately, 'standardization' by means of optical density does not guarantee a homogenous preparation, as various blood cell types probably contain varying amounts of the intrinsic ECL and fluorescent compounds (23, 24).

For comparison of cellular and fluid ECL fractions of tunicate 'blood', 1 ml of fresh *M. occidentalis* blood was centrifuged at 3.5 g for 10 min. Following centrifugation, 0.5 ml supernatant fluid was mixed with 0.5 ml TPA assay buffer (Igen Corp., Gaithersburg, MD, pH 7.5) and the cell pellet was resuspended in 1 ml TPA assay buffer. Then 0.5 ml of resuspended cells were mixed with 0.5 ml TPA assay buffer for ECL measurements.

Body fluid was difficult to extract from *S. plicata* and oysters (*Crassostrea virginica*) due to their tissue consistency. Thus, these animals were scrubbed in sea water and transferred to 75 ml of deionized water with 0.5% Triton X-100 and homogenized for 5 min in a Waring blender. Homogenates were allowed to settle for 15 min and the supernatant sample was decanted for ECL analyses. Five grams of colonial tunicates (*D. macdonaldi*) were removed from their substrates, washed in three changes of deionized water and ground with a mortar and pestle in 5 ml of deionized water plus 0.5% Triton X-100. *D. macdonaldi* samples were sedimented and supernatant fluid was used for ECL analysis. Processed samples were kept refrigerated for periods up to 1 month and frozen samples were kept for greater than 2 months without loss of ECL.

Blades of seagrass (*Thalassia testudinum*) were collected from some of the tunicate collection sites and were stripped of overlying marine organisms. Living (green) and dead (brown) terrestrial grass (*Eleusine indica*) was gathered from wooded areas around St Andrew Sound. Several blades of each plant species were crushed with a mortar and pestle in 5 ml deionized water and 0.5% Triton X-100 and the resultant green fluid was collected by suction. Absorbance readings at 600 nm were used to equalize sample concentrations.

Plankton were collected by towing a plankton net in St. Andrew Sound. Plankton were further isolated from lagoon water by concentrating ten-fold through a 0.45 μm millipore filter.

Table 1. Oxidation states of dissolved metal ions used in electrochemiluminescence studies

Metal	Oxidation State	Metal	Oxidation State
Ag	1+	Mg	2+
Al	3+	Mn	2+
As	3+	Na	1+
Au	1+	Ni	2+
Ba	2+	Pb	2+
Ca	2+	Re	3+
Cd	2+	Ru	2+
Co	2+	Sb	2+
Cr	6+	Se	4+
Cu	2+	Sn	2+
Eu	3+	Sr	2+
Fe	3+	Tb	3+
Ga	3+	Tl	1+
Hg	2+	W	4+
K	1+	V	5+
Li	1+	Zn	2+

Four drops of human blood were collected from an author (JGB) via a finger stick. Avian blood was collected via a heart puncture from a herring gull (*Larus argentatus*), immediately following death due to a natural injury. All blood samples were lysed in deionized water plus 0.5% Triton X-100 for ≥ 30 min with vigorous pipetting.

Bovine haematin was obtained from Aldrich Chemical Co. (Milwaukee, WI) and dissolved at 250 $\mu\text{g}/\text{ml}$ in 1 M KOH. Recombinant streptavidin (0.2 mg; GIBCO BRL, Life Technologies, Gaithersburg, MD) was labelled with 75 μg $\text{Ru}(\text{bpy})_3^{2+}$ -*N*-hydroxysuccinimide ester (Igen Corp.) in 100 μl dimethylformamide for 2 h at room temperature in the dark to mimic protein-bound ECL metal chelates. $\text{Ru}(\text{bpy})_3^{2+}$ -labelled streptavidin was purified on Sephadex G25.

Synthetic tunichrome analogues (*N,N',N''*-tris(2,3,4-trihydroxybenzoyl)-1,5,10-triazadecane(I); hereafter referred to as '2,3,4-tunichrome' and *N,N',N''*-tris(3,4,5-trihydroxybenzoyl)-1,5,10-triazadecane(II); hereafter referred to as '3,4,5-tunichrome'; 15) were the generous gifts of Dr Carl Carrano (Department of Chemistry, South-west Texas State University, San Marcos, TX). Synthetic tunichromes were dissolved in methanol and examined at a final concentration of 0.2 mg/ml with 100 ppm of a panel of dissolved metals in aqueous TPA buffer (1 ml total volume). Metal-tunichrome samples were gently mixed for 30 min at RT prior to ECL assessment.

Metal Ion Standards

Atomic absorption metal standards (1,000 ppm w/ dissolved in 2% HNO_3 or 10% HCl) were obtained from Aldrich, SPEX (Edison, NJ), and Fisher Scientific Corp. (Pittsburgh, PA). Oxidation states of the dissolved metal ions are listed in Table 1.

ECL assays

Typical ECL assays were performed by addition of 0.2 ml of biological samples to 12 \times 75 mm borosilicate glass tubes followed by addition of 0.1 ml of dissolved atomic absorption metal standard per tube (100 ppm final concentration) and 0.7 ml TPA assay buffer. In some experiments the concentration of metals was increased or decreased, but the biological sample volume was maintained at 0.2 ml and the overall sample volume was fixed at 1 ml by adjusting the TPA assay buffer volume. Measured pH values of biological sample-exogenous metal-TPA buffer mixtures varied between 5.0 and 7.0. In the case of tubes without exogenous metal ion additions, 0.8 ml of TPA assay buffer were added to 0.2 ml of biological sample.

ECL measurements of 25 $\mu\text{g}/\text{ml}$ bovine haematin (0.2 ml) and 7.5 $\mu\text{g}/\text{ml}$ streptavidin- $\text{Ru}(\text{bpy})_3^{2+}$ (0.1 ml) were made with 0.8 ml and 0.9 ml TPA assay buffer respectively. Tubes were vortex-mixed (100 rpm) on the Igen ORIGIN[®] analyser (commercial ECL sensor; 4-6,8,11) for periods of at least 5 min at RT. ECL intensity was quantitated by the ORIGIN[®] analyser, using an assay gain of 1,000, a linear ramp anodic excitation (5 V/sec) for 0.3 sec with a 1.5 V maximum excitation 'peak' waveform (standard default) and background ECL subtraction. Sample draw volume was 225 μl .

RESULTS

Previously frozen tunicate blood cell lysates taken from two different *M. occidentalis* animals and an oyster (*C. virginica*) homogenate demonstrated dramatically higher intrinsic ECL than human or avian blood, purified bovine haematin, concentrated phytoplankton (containing chlorophyll), or lagoon water taken from the tunicates' environment (Fig. 3). These results indicate that ECL compounds exist in the tunicates and oysters, which are not present in all types of biological samples.

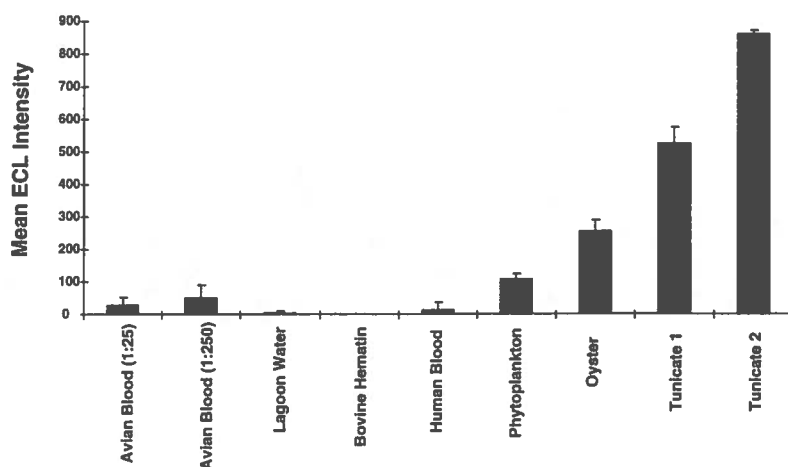


Figure 3. Comparison of intrinsic ECL from various biological and biochemical samples. The tunicate samples were from the lysed blood cells of two different previously frozen *M. occidentalis* animals. The avian blood was from a herring gull (*L. argentatus*). Means and standard deviations of three independent measurements are depicted

and probably do not originate from the marine environment (e.g. lagoon water and phytoplankton on which tunicates feed) unless environmental ECL compounds are greatly concentrated by tunicates and oysters. Fresh *M. occidentalis* body fluid samples did not require cell lysis to exhibit a relatively high degree of ECL, suggesting that ECL compounds are present both intracellularly and in fresh body fluid. The fact that avian (*L. argentatus*) blood gave a stronger ECL signal at the 1:250 vs. the 1:25 dilution indicates that overloading the ECL flow cell with more concentrated samples

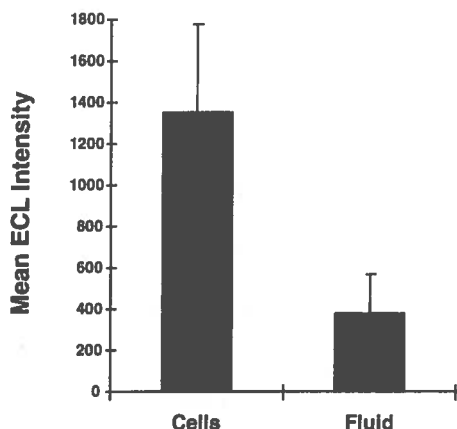


Figure 4. Comparison of ECL intensity of centrifugally separated *M. occidentalis* blood fractions. Means and standard deviations of five independent measurements are shown

can lead to sample self-absorption of ECL-generated photons. Such ECL self-absorption was observed routinely for tunicate and other biological samples assayed at high sample concentrations.

To further investigate the distribution of ECL between cellular and fluid fractions, *M. occidentalis* blood was centrifuged and the cell pellet was resuspended in TPA buffer so as to maintain the original cell concentration. Equal volumes of the cell suspension and fluid were used to ensure a valid ECL comparison. Fig. 4 illustrates the results of this experiment and indicates that most of the ECL activity resides in the cellular fraction although some ECL responsivity is present in the fluid phase. The cellular ECL correlates well with observations of extremely bright tunicate blood cell autofluorescence by epifluorescence microscopy made in this laboratory (data not shown) and in others (23, 24).

Fig. 5 summarizes the results of two ECL metal ion (oxidation states provided in Table 1) 'profiles' for previously frozen *M. occidentalis* blood cell lysates mixed with a final concentration of 100 ppm of 28 or 29 different exogenous metal ions. Overall evaluation of these data indicated that certain metals severely or completely inhibited the intrinsic ECL response. In general, the ECL inhibitory metal ions (e.g. Ag^+ , Au^+ , Cu^+ , Hg^{2+} , Sb^{2+}) are strong oxidants with highly positive standard reduction potentials (31). The strong oxidant nature of some exogenous metal ions may mean that they are capable of usurping high energy

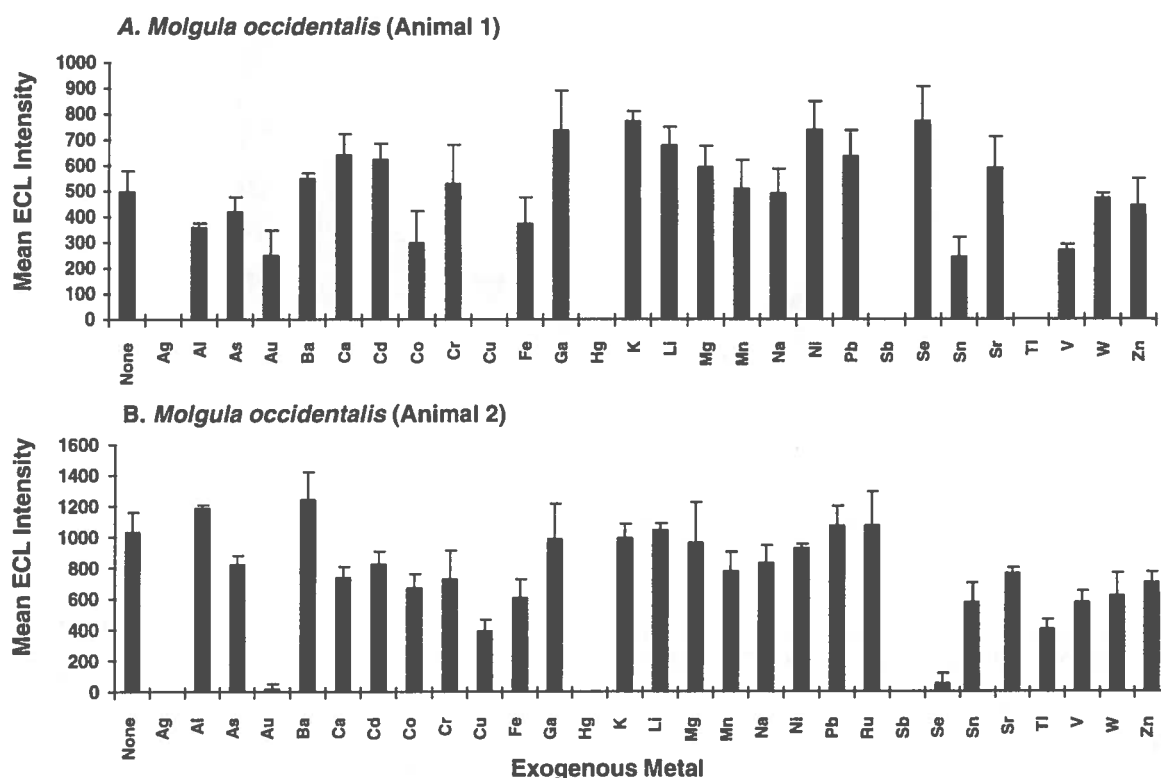


Figure 5. Comparison of ECL metal ion (100 ppm) profiles from the lysed blood cells of two different previously frozen *M. occidentalis* animals taken from the same location in St Andrew Sound. Means and standard deviations of three independent measurements are depicted

electrons from nascent $\text{TPA}^{+\bullet}$ radicals before the radical electrons can reduce the endogenous ECL compounds (Fig. 2; pathway II), resulting in reduced luminescence. No statistically significant evidence (by Student's *t*-test) of ECL enhancement due to the addition of various metal ions was observed, suggesting little presence of unbound aromatic ligands for complexation with exogenous metals (Fig. 2; pathway III). Differences in the ECL metal ion profiles of the two individual *M. occidentalis* animals depicted in Fig. 5 suggest intraspecies or individual differences in endogenous metal ion composition or overall biochemistry of the two animals.

To determine whether exogenous metal ion oxidants inhibit the ECL response of *M. occidentalis* blood cell lysates in a concentration-dependent manner, the concentration of metal ion oxidants was varied. Fig. 6 demonstrates a gradual shut-down of intrinsic ECL over a several log increase in the concentration of certain strong oxidizing exogenous metal ions. Fig. 6 also illustrates results

of attempts to influence ECL intensity by increasing the concentration of some metal ions beyond 100 ppm. Ru^{2+} (chosen because of its presence in the well known $\text{Ru}(\text{bpy})_3^{2+}$ ECL compound) was used along with Hg^{2+} and Sb^{2+} in these experiments. Ru^{2+} gradually decreased the ECL output in the 100–600 ppm range, while Hg^{2+} and Sb^{2+} continued to completely inhibit the ECL response.

The effect of Hg^{2+} on a model ECL system (i.e. $\text{Ru}(\text{bpy})_3^{2+}$ -labelled streptavidin) was also assessed and results are summarized in Fig. 7. The figure demonstrates that Hg^{2+} levels >10 ppm are required to inhibit the ECL response. This is a particularly interesting model system as the standard reduction potential of $\text{Ru}(\text{bpy})_3^{2+}$ is known to be highly positive (1.24 V) and the standard reduction potentials for reactions of Hg^{2+} are in the vicinity of 0.8–0.85 V (31). This illustrates that metals with lower standard reduction potentials can still interfere in a concentration-dependent manner with the ECL response of stronger oxidizing metal coordination complexes due to the ionic concentration dependence

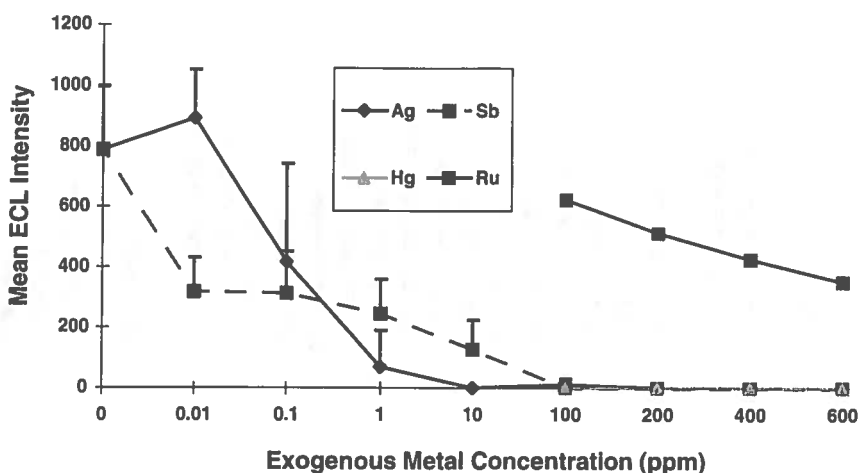


Figure 6. Effect of varying concentration of certain strong metal ion oxidants on intrinsic biological ECL of previously frozen blood cell lysates from *M. occidentalis*. Means and standard deviations of three independent measurements are depicted

of the overall redox potential (E) in the Nernst equation (32).

Comparison of Figs 5, 8 and 9 illustrates that there are differences in the ECL metal ion profiles of tunicates which are not strictly species-dependent as intraspecies differences between individual animals were again observed. Clearly, the data cannot be considered quantitative by the present methodology. Thus, only patterns and trends in ECL metal ion profiles are considered here. The predominant pattern that appears to emerge from Figs 5, 8 and 9 is that strong metal ion oxidants drastically or completely inhibited intrinsic tunicate ECL.

It also appears that the individual physiological state of animals taken from the same environment

may play an important role in the redox state of their tissues or the collection of metals and organic ligands that individuals contain. This is illustrated by comparison of Fig. 9A with Fig. 10, which demonstrates dramatic differences in ECL metal ion profiles between a freshly processed tunicate and 13-day starved animal of the same species (*S. plicata*) taken from a location very near the animal represented in Fig. 9A. Not only did starvation lead to a large reduction in body mass, but the overall ECL intensity was drastically reduced and changes in the degree of ECL inhibition by various exogenous metal ions were apparent. For example, Ag^+ , Au^+ , Hg^{2+} , Tl^+ and V^{5+} completely inhibited intrinsic ECL in the starved, but not in the fresh, tunicate taken from the same area of the lagoon.

Fig. 11 demonstrates that an intrinsic ECL metal ion profile (abbreviated to only 14 exogenous metal ions) from an oyster homogenate differed from any of the previous tunicate ECL metal ion profiles. For example, Ru^{2+} completely suppressed the oyster, but not the tunicate, ECL response. This observation further suggests that differences in the metallic or biochemical composition of various marine animals may account for differences in the observed ECL metal ions profiles.

ECL from natural chlorophyll (28) and synthetic porphyrin-like molecules has been observed (29, 30). Therefore, it was postulated that plant materials might exhibit noteworthy intrinsic ECL. Fig. 12 demonstrates that live (green) grass (*E. indica*) extracts exhibited high solution-phase ECL levels

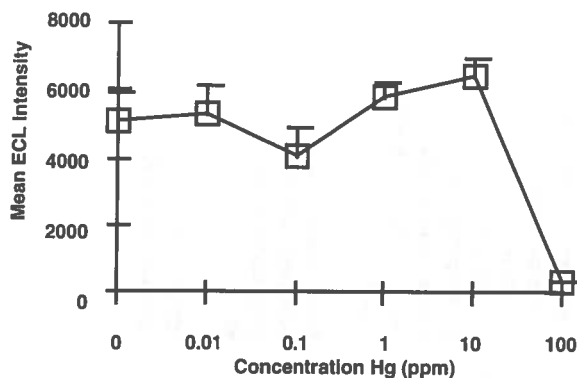


Figure 7. Effect of varying dissolved Hg^{2+} concentration on the ECL of $7.5 \mu\text{g/ml}$ streptavidin- $\text{Ru}(\text{bpy})_3^{2+}$. Means and standard deviations of three independent measurements are shown

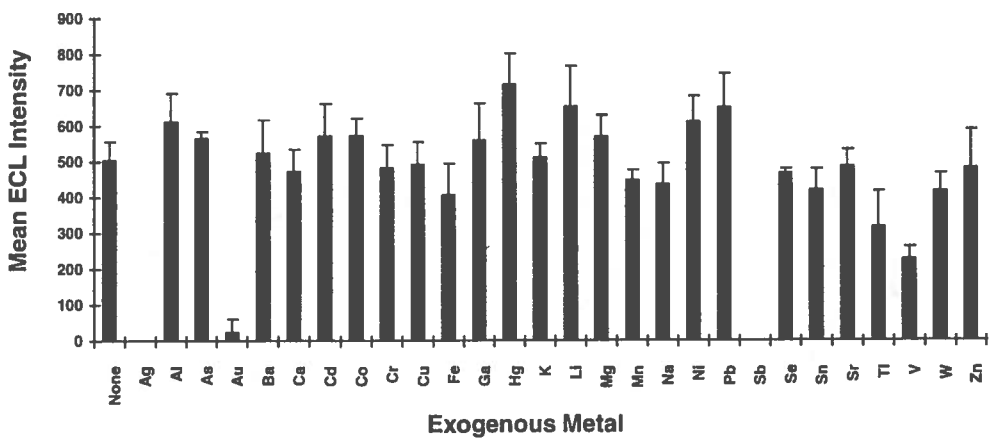


Figure 8. ECL metal ion (100ppm) profile of a reddish colonial tunicate (*D. macdonaldi*) fluid extract. Means and standard deviations of three independent measurements are given

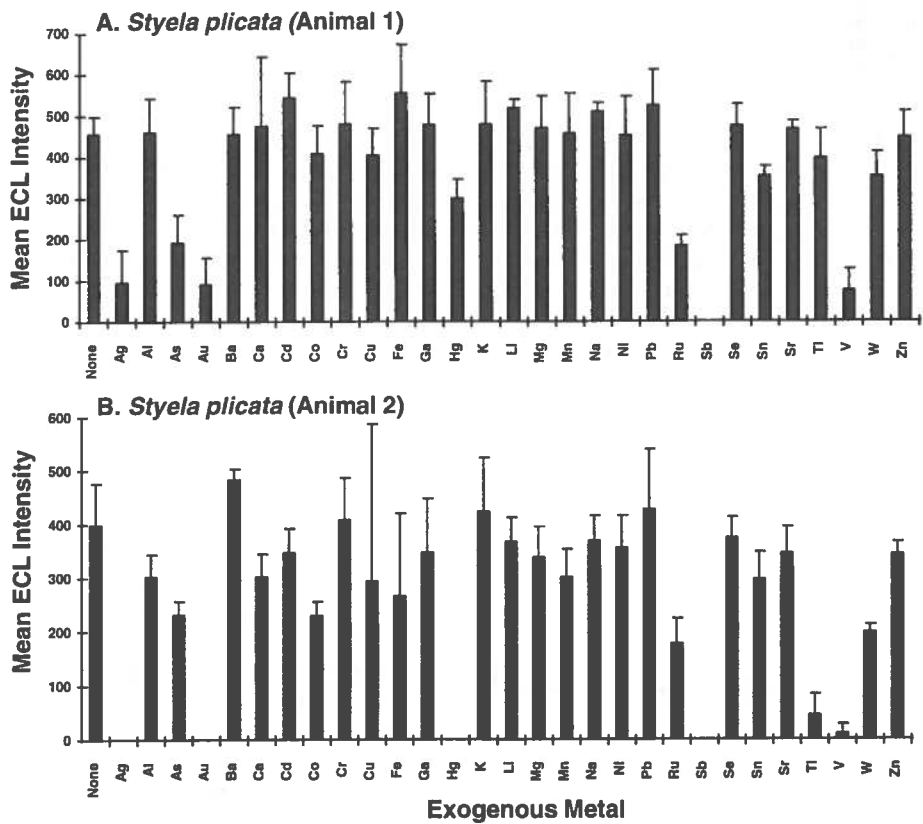


Figure 9. ECL metal ion (100ppm) profile of fluid extracts from two different homogenized *S. plicata* animals from different areas in St Andrew Sound. Means and standard deviations of three independent measurements are depicted

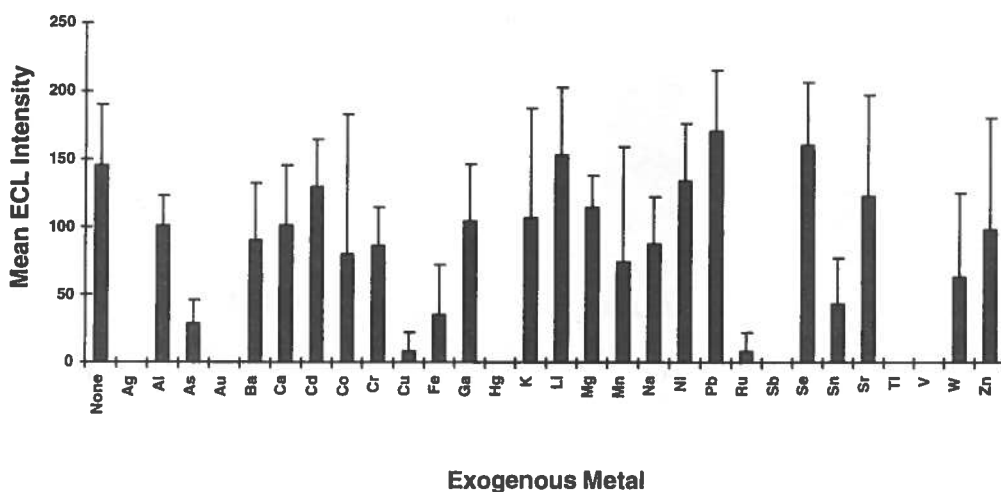


Figure 10. ECL metal ion (100 ppm) profile of fluid extracts from a 13-day starved, homogenized *S. plicata* animal taken from the same location in St Andrew Sound as the animal represented in Fig. 9A. Means and standard deviations of three independent measurements are depicted

that were not present in identically processed dead (brown) grass of the same species. Seagrass (*T. testudinum*), which is normally covered with various flora and fauna, but remains deep-green colored, exhibited a relatively strong, but lower, ECL response (Fig. 12). Comparison of abbreviated (8 exogenous metal ions) ECL profiles (Fig. 13) suggests differences in the biochemistry of terrestrial and seagrasses as well.

Fig. 14 summarizes the ECL results of synthetic tunichrome isomers (Fig. 1) in the presence and absence of added metal ions. The 2,3,4-tunichrome demonstrated some intrinsic ECL capability, which appeared to be enhanced by the addition of various metal ions such as Ag^+ , Au^+ , Cd^{2+} , Hg^{2+} , Se^{4+}

and V^{5+} . Of these metals, however, only Au^+ showed a statistically significant (Student's *t*-test) enhancement over baseline ECL. Similarly, the 3,4,5-tunichrome showed a statistically significant ECL enhancement by interacting with Hg^{2+} . Unlike the 2,3,4-tunichrome, the 3,4,5-tunichrome had no detectable inherent ECL and demonstrated no significant ECL enhancements with other metal ions except Hg^{2+} .

DISCUSSION

ECL has been described for different types of molecules and by different mechanisms (1-4).

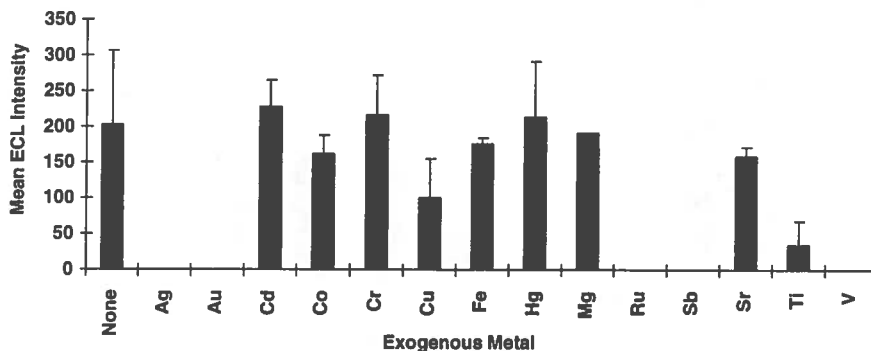


Figure 11. ECL metal ion (100 ppm) profile of a fluid extract from a homogenized oyster (*C. virginica*) mussel. Means and standard deviations of three independent measurements are shown

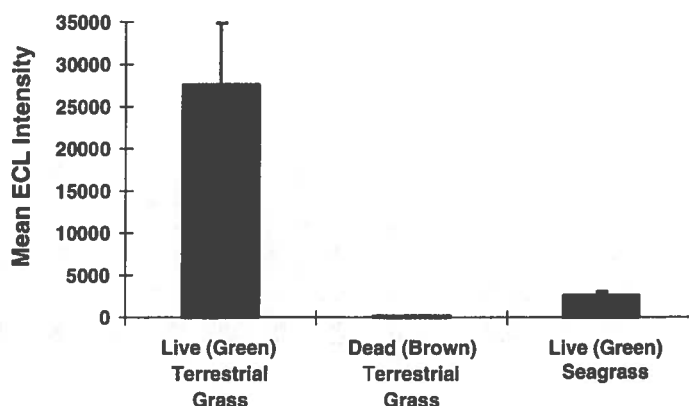


Figure 12. Comparison of intrinsic biological ECL from live (green) and dead (brown) terrestrial grass (*E. indica*) and live seagrass (*T. testudinum*). Means and standard deviations of three independent measurements are shown

Organic-metal complexes are only one of several classes of molecules capable of ECL (1). Most studies of ECL have employed synthetic ECL compounds or plant materials such as chlorophyll (28). Thus, the current report probably represents the first description of noteworthy solution-phase ECL induced in samples of marine animal origin, although Yu and Bruno (6) observed elevated

background ECL from freshwater fish samples in a study of immunomagnetic-ECL detection of pathogens in foods.

Biological ECL phenomena are significant for two reasons. First, ECL was not strongly inducible in all animal preparations examined (e.g. human and avian blood), thus the more efficient intrinsic ECL compounds appear to be peculiar to certain

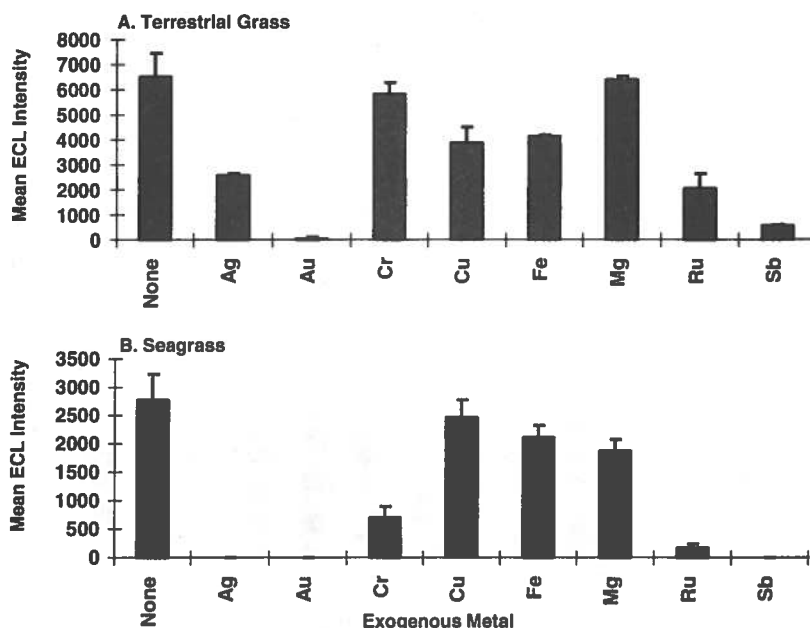


Figure 13. ECL metal ion (100 ppm) profiles of fluid extracts from terrestrial grass (*E. indica*) and seagrass (*T. testudinum*). Means and standard deviations of three independent measurements are depicted

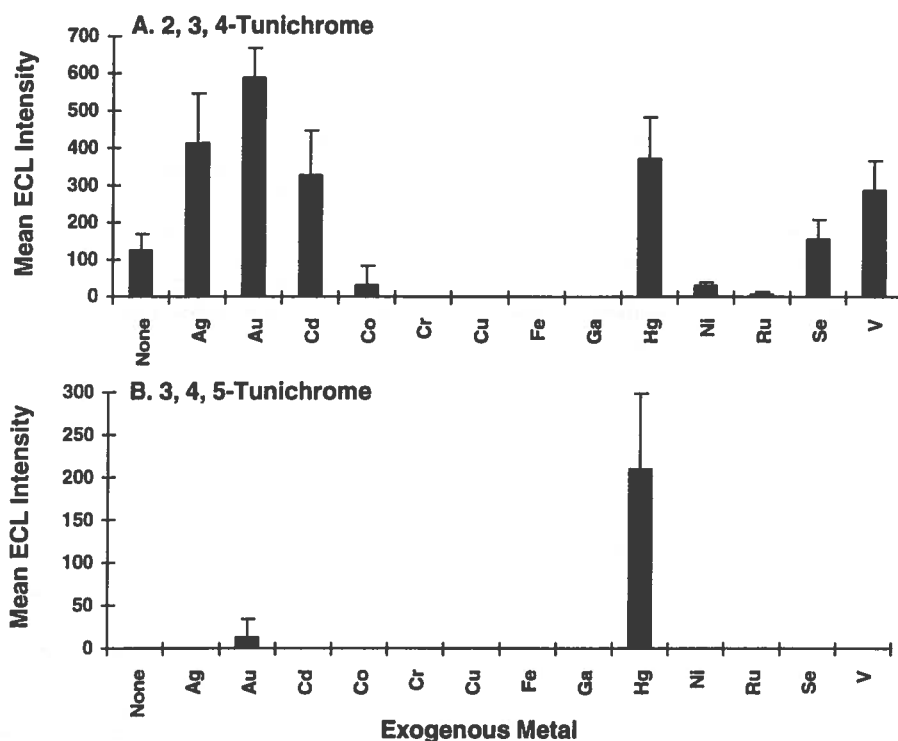


Figure 14. Synthetic tunichrome isomer (depicted in Fig. 1) ECL in the presence and absence of added metal ions (100 ppm). Means and standard deviations of three independent measurements are given.

types of organisms. Second, biologically derived ECL compounds may serve as easily extractable sources of ECL labels with, perhaps, novel properties (spectral or lifetime characteristics) for various molecular probes (e.g. immunochemical and nucleic acid-based probes).

Another potentially significant finding stems from the observation that intrinsic ECL was susceptible to near or complete inhibition by a variety of metal ion oxidants. This may be due to usurping of free radical electrons from $\text{TPA}^{+\bullet}$, but other mechanisms involving the 'soft acid' nature of various metal ions to preferentially complex with sulphur-containing or other ligands could lead to quenching of ECL, as could replacement of resident metal ions in extant coordination complexes. In short, a number of unknown redox reactions involving metals or non-metals at the anodic surface may play a role in the observed ECL inhibitions of such biochemically complex systems.

Regardless of the mechanism and active species, however, differences in the tunicate ECL profiles resulting from exposure to various exogenous metal ions appeared to be somewhat dependent

on individual biochemical variability or the physiological state of the organism. This quality may make the technique valuable as a tool for biochemical studies. The intraspecies (individual) differences may be due to differences in age, the microenvironments from which specimens were collected (33), despite collection of animals proximal to one another, or other physiological factors. Seasonal variations in the biochemistry of tunicates have been reported (13, 34), but can probably be ruled out as a source of variation in the present work as intraspecies differences in ECL profiles were observed from animals harvested on the same day.

The ECL metal ion profiles presented here may be thought of as a crude means of varying the redox environment to modify ECL light output. Generation of such profiles was tedious. It would be much simpler to create an ECL flow cell in which the electrode voltage could be varied and the ECL intensity could be gleaned as a function of electrode potential. In this way, a voltage scan of the sample could be performed creating the ECL analogue to an amperometric voltammogram (11, 32). This was not possible with the

current ORIGEN[®] analyser configuration. Such 'ECL-potentiometry' could represent a powerful new tool for biochemical studies of ECL-compent organisms or tissues which might be used to reveal subtle differences in the metallic or biochemical composition of specimens. If coupled to ECL spectra and lifetime analyses, an even more powerful analyser might result.

At present, the intrinsic ECL remains phenomenological. However, it is reasonable to postulate that organic-metal coordination complexes might be responsible for the observed ECL, since tunicates and oysters, which are known for their metals accumulation, demonstrated fairly strong intrinsic ECL. Moreover, tunicates are known to produce tunichrome blood pigments which may chelate, but more likely reduce (20), metal ions. It is interesting to note that tunicates are known for their tremendous ability to concentrate Fe and V from seawater, yet neither of these metals greatly enhanced the baseline ECL of crude tunicate extracts or synthetic tunichromes. Tunicates possess a variety of unusual biochemicals, such as cyclopeptides (14, 22) and high levels of the amino acid taurine (34). Therefore, it is also reasonable to hypothesize that tunicates could produce an entirely organic and perhaps novel ECL compound.

Certain metalloporphyrins were analysed for ECL, either directly or indirectly in the present study. Interestingly, isolated bovine haematin was not an ECL compound, but chlorophylls from plant extracts emit ECL. This suggests that Fe^{3+} (in haemes) probably quenches the ECL response, while Mg^{2+} (in chlorophylls) does not.

The efficiency of observed tunicate ECL cannot be evaluated at present, but it may be relatively high, since solutions of $\text{Ru}(\text{bpy})_3^{2+}$ -labelled streptavidin produce much lower ECL intensities when measured in the solution-phase mode, rather than when bound to magnetic beads on the electrode surface in a sandwich immunoassay format (4–6). The ECL sensor used is designed for use with magnetic beads to concentrate $\text{Ru}(\text{bpy})_3^{2+}$ at the anode surface where the electric field is strongest (4–6). Thus, solution phase measurements for any ECL compound, while valid, will be weaker than similar measurements made with magnetic bead-mediated concentration at the electrode surface.

There are many phenomena surrounding the observed biological ECL reported here, such as which ECL compound or compounds are present in samples and why these molecules or compounds

are able to exhibit ECL in neutral or slightly acidic aqueous solutions (1). At present, the only certain aspects of the observed biological ECL are that (a) it is at least anodically induced (cathodic ECL has not been investigated); (b) it is inhibited by exogenous metal ion oxidants in a dose-dependent fashion. Finally, it is clear that some synthetic tunichrome-metals complexes can produce weak ECL in isolated *in vitro* systems. Whether tunichromes associate with metals to produce ECL compounds in the more complex *in vivo* situation remains unknown. It is clear, however, that the isolated biochemical systems, such as the tunichrome-metal systems, differ in their ECL behaviour from that of the crude marine animal extracts.

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